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Macro Creatine Kinase BB: Evidence for Specific Binding between Creatine Kinase BB and Immunoglobulin G

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Dedicated to Professor Dr. J. Bierich on occasion of his 60th birthday

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Summary: In the sera of four female patients with proven IgG-linked creatine kinase BB (macro creatine kinase BB) we studied the nature of the binding between creatine kinase isoenzymes and immunoglobulin G. The specificity towards the isoenzyme bound, the recombination of the complex after treatment with acid buffer, the site of the binding between both partners and an apparently monoclonal nature of the immunoglobulin G involved in the complex indicate that a specific antigen-antibody reaction is responsible for the existence of macro creatine kinase BB in the sera investigated.

Makro-Kreatinkinase-BB: Nachweis einer spezifischen Bindung zwischen Kreatinkinase-BB und Immunglobulin G

Zusammenfassung: In den Seren von vier Patientinnen mit nachgewiesener IgG gebundener Kreatinkinase-BB (Makro-Kreatinkinase-BB) untersuchten wir die Bindung zwischen den Kreatinkinase-Isoenzymen und Immunglobulin G. Diese enzymbindenden Immunglobuline sind durch ihre Spezifität charakterisiert und zeichnen sich durch ihre Rekombinationsfähigkeit zu Makro-Kreatinkinase aus. Die Lokalisation der Bindung auf den Fab-Fragmenten und die offensichtlich monoclonale Herkunft der bindenden Immunglobuline machen eine Antigen-Antikörper-Reaktion als Ursache dieser Makro-Kreatinkinase-BB wahrscheinlich.

Introduction

Using exclusion chromatography, macro creatine kinase (1, 2) (Creatine kinase, ATP: creatine-phosphotransferase, EC 2.7.3.2) was found in the sera of patients with an unusually high residual creatine kinase activity following immunological inhibition (3) of creatine kinase M[1]) subunits. More detailed investigations showed the presence of at least two different types of macro creatine kinases (4). In our cases of macro creatine kinasaemia type 1 we have been able to classify the macro creatine kinase as a complex between immunoglobulin G and the isoenzyme creatine kinase BB (5). Up to now it has remained obscure whether these complexes of immunoglobulin with enzyme represent specific antigen-antibody complexes or non-immunological complexes. In this paper we present proof of an obvious specific immunological reaction between creatine kinase BB and the serum antibodies.

[1]) Abbreviations: creatine kinase BB, creatine kinase MB, creatine kinase MM: brain type, heart type, striated muscle type creatine kinase isoenzymes; IgG: immunoglobulin G; $F(ab')_2$, Fab: antibody binding fragments of immunoglobulin G; Fc: crystallisable fragment of IgG; Tris: tris(hydroxymethyl)methylamine; DEAE: diethylaminoethyl.

Material and Procedures

Enzyme assay

Creatine kinase activity was measured at 25 °C or, if higher sensitivity was necessary, at 37 °C with an N-acetylcysteine reactivated method (6) (CK-NAC: Boehringer, Mannheim, FRG, No: 126357 or E. Merck, Darmstadt, FRG, No: 14109, 14110 and 14111). The activity of our isoenzyme preparations was assayed with a photometer Eppendorf (M 1101). The post-column creatine kinase activities of the chromatographic runs were determined with an ACP 5040 analyzer (Eppendorf Gerätebau, Hamburg, FRG). After electrophoresis the activity of the creatine kinase isoenzymes was visualized as described elsewhere (5) and scanned (Liposcript, Hirschmann, Unterhaching, FRG. Filter: BG 18, 510 nm).

Isoenzyme purification

Human skeletal muscle, heart muscle and uterus, obtained at autopsy or surgery were used as tissue sources of creatine kinase MM, MB and BB. All tissues were homogenized in Tris buffer (1 mmol/l, pH 8.0). The extracts were clarified by centrifugation (Sorvall RC 2, DuPont, Wilmington, USA) at 4 °C and 30000 *g* for 20 minutes and the resulting supernates (10 ml) were applied to a 22 × 0.9 cm column of DEAE-Sepharose CL-6B equilibrated with the homogenizing buffer. The isoenzymes were eluted by a linear gradient: "weak buffer": 1 mmol/l Tris, pH 8.0, "strong buffer": 50 mmol/l Tris, 300 mmol/l NaCl, pH 7.0. The appropriate fractions were pooled, concentrated and ultrafiltered with Tris buffer (50 mmol/l, 50 mmol/l NaCl, 20 mmol/l N-acetylcysteine, pH 7.0) in an Amicon concentrator

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(membrane UM 10; Amicon, Witten, FRG). The purity of the creatine kinase isoenzymes was checked by electrophoresis on agarose gel (5).

^{125}I labeled creatine kinase BB was purchased from DRG instruments (Marburg, FRG). Impurities were removed by ion exchange chromatography using DEAE-Sephadex A-50 columns (Roche Diagnostics, Nutley, USA).

Purification of Immunoglobulin G

Immunoglobulin G (1, 2, 4-subclass) from the sera (4 ml) of four patients (Doe. E., Kes. E., Rud. M., Sch. L.) with proven (according to (5)) IgG-linked creatine kinase BB, was purified by affinity chromatography on Protein A-Sepharose CL-4B (5 X 0.8 cm) (7, 8). IgG was eluted with an acid glycine buffer (50 mmol/l, pH 3.0). The IgG containing fractions were pooled, concentrated (membrane UM 10) to 4 ml and the pH was adjusted to 7.0 with NaOH (1 mol/l).

Recombination of macro creatine kinase in vitro

The purified IgG solutions were incubated with creatine kinase BB or MB for 1 h at 4 °C before electrophoresis, activity staining and scanning.

Cross reactivity between IgG and creatine kinase BB or MB was tested by incubating 120 µg of immunoglobulin G and 3 mU of creatine kinase MB with increasing amounts of creatine kinase BB (0–3 mU). After incubation for 1 h at 4 °C the samples were electrophoresed, stained for creatine kinase activity and photographed.

Pepsin digestion

4 ml of the IgG solution and 0.5 mg pepsin (EC 3.4.23.1) (Merck, Darmstadt, FRG, No. 7192) were incubated at 37 °C for 17 h (50 mmol/l glycine-HCl buffer, pH 4.5). Digestion was stopped by cooling and adjusting the pH to 8.0 with NaOH (1 mol/l).

Papain digestion

4 ml of the IgG solution and 1.2 mg of papain (EC 3.4.22.2) (Merck, Darmstadt, FRG, Nr. 7144) were incubated according to *Goding* (8) for 6 h at 37 °C and pH 8.0. The reaction was stopped by cooling the mixture to 0 °C.

Preparation of F(ab')$_2$ fragments

After pepsin treatment of the IgG, undigested IgG was removed by affinity chromatography on Protein A-Sepharose CL-4B (fig. 1a). The crude F(ab')$_2$ fraction was further purified by exclusion chromatography on Sephadex G-100 sf (59 X 0.9 cm) (fig. 1b). Fractions 39–50 were pooled and concentrated (membrane UM 10) to 0.8 ml. The molecular weight was confirmed to be in the range of 95000 (fig. 2, C), and the purity of the F(ab')$_2$ fragments was checked by antisera using the *Ouchterlony* technique.

Preparation of Fab fragments

After papain treatment of the IgG, undigested IgG and Fc fragments were removed by affinity chromatography on Protein A-Sepharose CL-4B. The crude Fab fraction was further purified by exclusion chromatography on Sephadex G-100 sf (59 X 0.9 cm). Fractions 46–59 were pooled and concentrated (membrane UM 10) to 0.8 ml. The purity was checked by rechromatography as described above (fig. 1c). The molecular weight was determined to be approximately 42000 (fig. 2, A).

Preparation of Fc fragments

After papain digestion of the IgG, the undigested IgG and the Fc fragments remained on the column during affinity chromatography on Protein A-Sepharose CL-4B. They were eluted together at pH 3.0 (glycine-HCl buffer, 50 mmol/l). Further purification was achieved by exclusion chromatography on Sephadex G-100 sf (59 X 0.9 cm). Fractions 47–58 were pooled and concentrated to 0.8 ml (membrane UM 10); their purity was checked

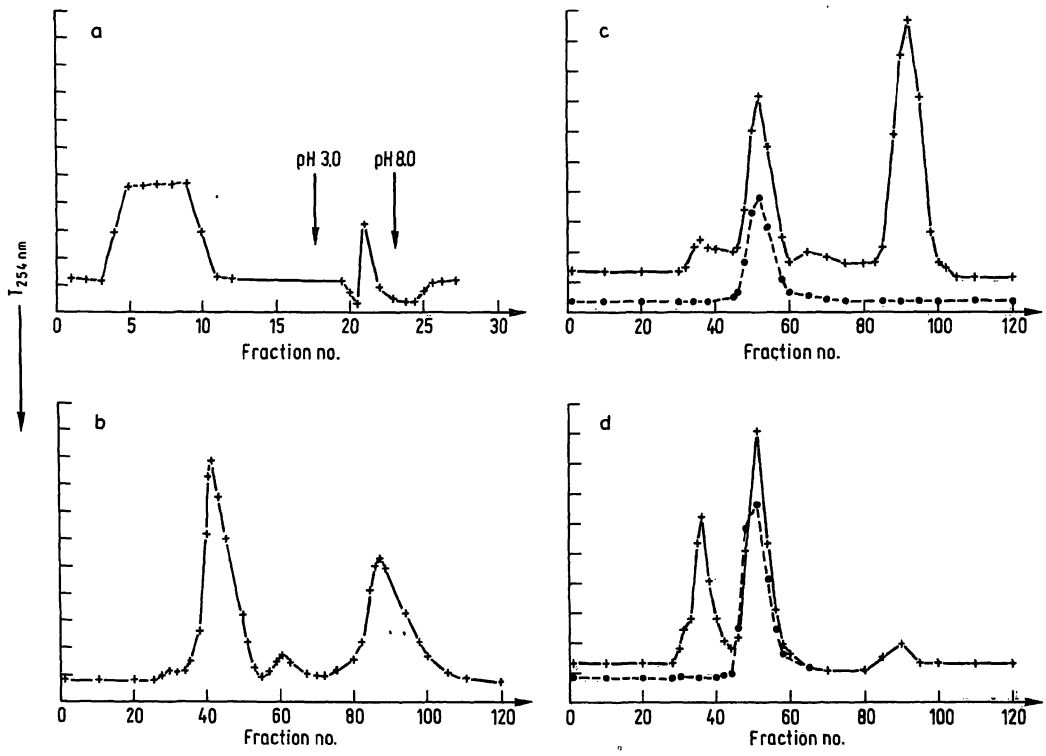

Fig. 1. Purification of IgG fragments after pepsin/papain digestion.

Fig. 1a. Affinity chromatography on Protein A-Sepharose CL-4B of the reaction mixture after pepsin digestion of IgG; 2 ml fractions. Fractions 4–10: crude F(ab')$_2$ fragments. Fraction 21: undigested IgG. y-axis: Transmission at 254 nm.

Fig. 1b–1d. Exclusion chromatography on Sephadex G-100 sf (59 X 0.9 cm) of F(ab')$_2$ (fig. 1b), Fab (fig. 1c) and Fc (fig. 1d) fragments; 440 µl fractions.

+—+—+: crude fragments. •—•—•: purified fragments. y-axis: Transmission at 254 nm.

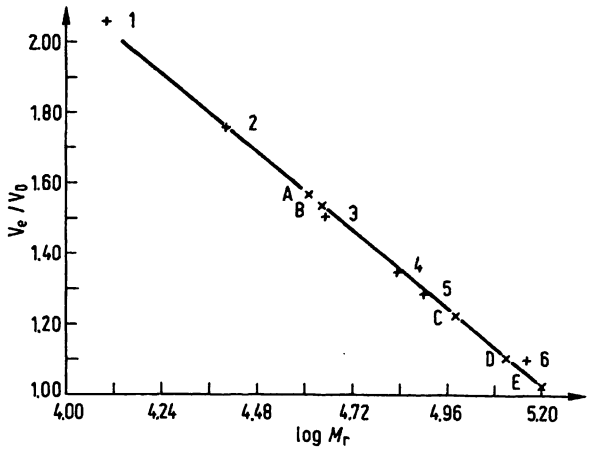

Fig. 2. Determination of molecular weights by exclusion chromatography on Sephadex G-100 sf (59 × 0.9 cm).

Calibration standards (+)
1 Cytochrome c (12500)
2 Chymotrypsinogen A (25000)
3 Albumin from hen egg (45000)
4 Albumin from bovine serum (68000)
5 Creatine kinase (CK) BB (80000)
6 IgG (146000) (10).

Results (x)

A: Fab fragments	M_r: 42000 (41000) (9)
B: Fc fragments	M_r: 45000 (50000) (9)
C: $F(ab')_2$ fragments	M_r: 95000 (92000) (9)
D: CK-Fab complex	M_r: 130000 (expected: 121000)
E: CK-$F(ab')_2$ complex	M_r: at least 160000

by rechromatography (fig. 1d). The molecular size was estimated to be approximately 45000 (fig. 2, B).

Determination of the binding site between creatine kinase B subunits and immunoglobulin G or its fragments

200 µl of IgG or its fragments and 67 µl of creatine kinase BB (2100 U/l) or creatine kinase MB (2000 U/l) were incubated for 1 h at 25 °C and 1 h at 4 °C (pH 7.0).

150 µl of these mixtures were chromatographed on Sephacryl S-200 sf (55 × 0.9 cm) or Sephadex G-100 sf (59 × 0.9 cm). Post column creatine kinase activities were determined as described. 12 µl of each mixture were applied onto agarose plates, electrophoresed and stained for creatine kinase activity. 20 µl of the mixtures were used in the double diffusion method (*Ouchterlony*) (5). The antisera applied were purchased from Dako, Køpenhavn, Denmark (Anti-IgG (Fc specific), Anti-kappa, Anti-lambda, Anti-IgG (gamma chain)) and Behringwerke, Marburg, FRG (Anti-IgG/Fd, Anti-IgG/Fab, Anti-IgG/Fc).

10 µl of IgG or its fragments and 10 µl of ^{125}I-labeled creatine kinase BB were incubated at 25 °C for 1 h. Electrophoresis was performed as described and the plates were placed on X-ray films for autoradiography.

Results

Immunoglobulin G was easily separated from the sera by affinity chromatography on Protein A-Sepharose CL-4B. As already described (5, 11) not only free immunoglobulin G but also enzyme-linked immunoglobulin G is quantitatively fixed by Protein A. Acid elution of the immunoglobulin G resulted in a pure immunoglobulin G fraction, which no longer showed creatine kinase activity.

Immunoglobulin G from all four patients' sera recombined not only with autologous creatine kinase BB (Doe. E.) (5), but also with homologous creatine kinase BB, to form macro creatine kinase. In one case (Rud. M.), after incubation of immunoglobulin G with creatine kinase MB, macro creatine kinase MB was formed in amounts comparable to the formation of macro creatine kinase BB from creatine kinase BB and immunoglobulin G. The immunoglobulin molecules of this serum showed a marked cross reactivity between creatine kinase BB and creatine kinase MB, as determined by incubating constant amounts of immunoglobulin G and creatine kinase MB with increasing amounts of creatine kinase BB. Figure 3 shows the displacement of creatine kinase MB from its immunoglobulin G linkage due to the addition of creatine kinase BB. Creatine kinase MM never became fixed to immunoglobulin G in measurable amounts. Figure 4 shows densitograms after electrophoresis of the reconstituted macro creatine kinase BB and macro creatine kinase MB.

After papain digestion, the purified Fab fragments revealed a high affinity to creatine kinase BB, which resulted in the formation of complexes between Fab and creatine kinase BB. One example (Rud. M.) is given in figure 5a. The shift of the molecular weight of the creatine kinase activity to higher values due to complexation with Fab fragments is clearly documented by exclusion chromatography. From these data we concluded that complexes with a 1:1 molar ratio between the Fab fragments and creatine kinase BB are formed. The molecular mass of these complexes was 130000 (fig. 2, D). The formation of atypically migrating creatine kinase activity bands during electrophoresis of the Fab-creatine kinase BB mixture also indicated stable complex formation (fig. 4).

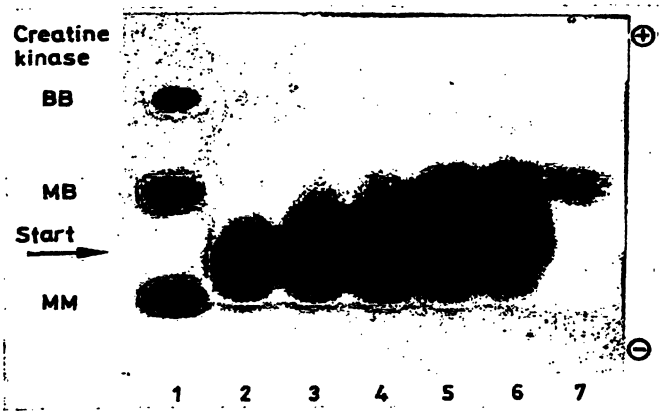

Fig. 3. Macro creatine kinase MB: displacement of bound creatine kinase MB by increasing amounts of creatine kinase BB. Electrophoresis on agarose gel; activity staining.
→: electrophoretic origin. Anode at the top.
1: standard, 2–6: macro creatine kinase MB (3 mU creatine kinase MB) incubated with increasing amounts of creatine kinase BB (0, 0.6, 1.2, 2.4, 3.0 mU). 7: creatine kinase MB.

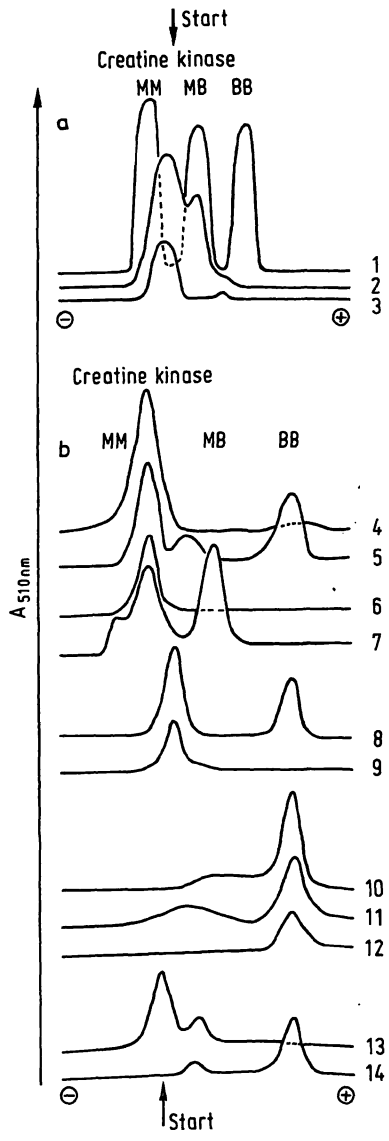

Fig. 4. Agarose gel electrophoresis, scanned at 510 nm.
→: electrophoretic origin
Stained for enzyme activity: lanes 1–3, 6, 7, 12
Autoradiograms: lanes 4, 5, 8–11, 13, 14
y-axis: Absorbance at 510 nm.

Fig. 4a.

1: standard: creatine kinase MM, creatine kinase MB, creatine kinase BB (left to right)
2: macro creatine kinase MB, recombined (IgG Rud. M. and creatine kinase MB)
3: macro creatine kinase BB, recombined (IgG Rud. M. and creatine kinase BB)

Fig. 4b.

4: $F(ab')_2$-^{125}I-CK-BB (Kes. E.)
5: $F(ab')_2$-^{125}I-CK-BB (Doe. E.)
6: $F(ab')_2$-CK-BB (Rud. M.)
7: $F(ab')_2$-CK-MB (Rud. M.)
8: Fab-^{125}I-CK-BB (Kes. E.)
9: Fab-^{125}I-CK-BB (Rud. M.)
10: Fc-^{125}I-CK-BB (Doe. E.)
11: Fc-^{125}I-CK-BB (Kes. E.)
12: Fc-CK-BB (Rud. M.)
13: ^{125}I-macro creatine kinase BB
14: ^{125}I-CK-BB

Under the same conditions no similar high level of complex formation was detectable after incubation of purified Fc fragments and creatine kinase BB (fig. 4, 5b).

After pepsin digestion the purified $F(ab')_2$ fragments showed a high affinity to creatine kinase BB, which resulted in the formation of complexes. Two examples (Kes. E., Doe. E.) are given in figure 5 a and 5 c. The shift of the molecular weight of the creatine kinase activity to higher values due to complexing with the $F(ab')_2$ fragments is clearly demonstrated by exclusion chromatography. From these data we concluded that complexes with a 1:1 or 2:1 molar ratio between creatine kinase BB and the $F(ab')_2$ fragments are formed. The molecular mass of the $F(ab')_2$-creatine kinase BB complexes was at least 160000 (fig. 2,E): a value which already almost equals the void volume of our G-100 sf column. Furthermore, the formation of atypically migrating creatine kinase activity bands after electrophoresis, due to complexing of enzyme with the $F(ab')_2$ fragments, is documented in figure 4.

In the sera of two patients (Rud. M., Kes. E.) we determined the light chains of the creatine kinase BB and MB-binding immunoglobulins. Figure 6 shows our results on the $F(ab')_2$ fragments of serum Rud. M. after double diffusion (*Ouchterlony*) and staining for creatine kinase activity. The two main precipitin lines indicated binding between creatine kinase BB or (but to a lesser degree) creatine kinase MB and kappa-type light chains in the $F(ab')_2$ region of the immunoglobulin G molecule. In addition, the faint precipitin lines between anti-Fd serum and the $F(ab')_2$-linked creatine kinase isoenzymes and the absence of precipitin lines with anti-Fc serum demonstrated pure $F(ab')_2$ fragments.

In serum Kes.E. the creatine kinase-binding immunoglobulin G light chains proved to belong to the lambda subclass.

A summary of our results is given in table 1.

Discussion

Immunoglobulin G-linked creatine kinase BB isoenzyme has been reported in the sera of patients with several diseases. Up to now it has remained unclear whether this is a specific antigen-antibody reaction or a nonspecific protein-immunoglobulin complex (1, 2, 12). Here we show the existence of an antibody for creatine kinase BB. The immunoglobulin G-linked creatine kinase BB in the four sera investigated fulfilled the following criteria which are typical for specific immunological antigen-antibody reactions:

1. The binding between creatine kinase BB and immunoglobulin G is reversible: Acidolytic treatment of the macro creatine kinase complexes destroyed all creatine kinase activity. After chromatographic purification, the acid-stable immunoglobulin G fractions easily recombined not only with autologous but also with homologous creatine kinase BB, to form macro creatine kinase. Furthermore these results indicate that the formation of

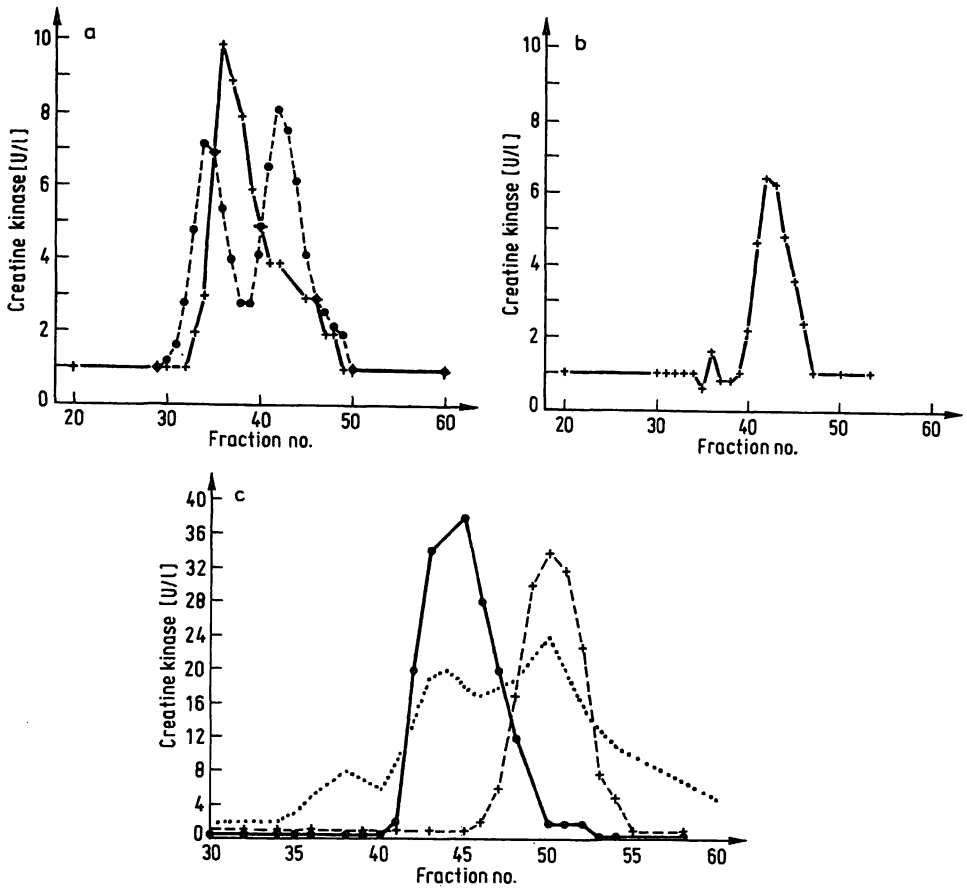

Fig. 5. Complexation of creatine kinase BB by fragments of IgG.

Fig. 5a. Exclusion chromatography on Sephadex G-100 sf (59 X 0.9 cm), 440 µl fractions
+—+—+: Fab (Rud. M.) incubated with creatine kinase BB.
●—●—●: $F(ab')_2$ (Kes. E.) incubated with creatine kinase BB.
First peak: $F(ab')_2$-CK-BB complex, second peak: normal creatine kinase BB.

Fig. 5b. Same column as fig. 5a, 440 µl fractions
+—+—+: Fc (Doe. E.) incubated with creatine kinase BB.

Fig. 5c. Exclusion chromatography on Sephacryl S-200sf (52 X 0.9 cm), 440 µl fractions
·····: Transmission at 254 nm, normal serum
+—+—+: Creatine kinase BB in normal serum (creatine kinase activity)
●—●—●: $F(ab')_2$ (Doe. E.) incubated with creatine kinase BB.

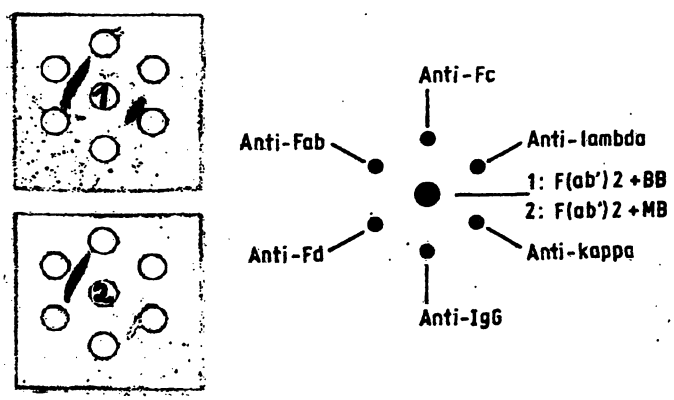

Fig. 6. Determination of the binding of creatine kinase BB and creatine kinase MB with $F(ab')_2$, and determination of the IgG light chains complexing creatine kinase BB and creatine kinase MB. *Ouchterlony* technique on agarose plates. Creatine kinase activity staining.

Tab. 1. Complexing of creatine kinase (CK) isoenzymes by immunoglobulin G or its fragments from the sera of four patients with proven macro creatine kinase BB.

Patient	Complexing with					
	CK-BB		CK-MB		CK-MM	
Doe.E.	IgG:	+	IgG:	trace	IgG:	-
	$F(ab')_2$:	+				
	Fab:	+				
	Fc:	trace				
Kes.E.	IgG(λ):	+	IgG:	-	IgG:	-
	$F(ab')_2$:	+				
	Fab:	+				
	Fc:	trace				
Rud.M.	IgG(κ):	+	IgG(κ):	+	IgG:	-
	$F(ab')_2$:	+	$F(ab')_2$:	+		
	Fab:	+				
	Fc:	-				
Sch.L.	IgG:	+	IgG:	-	IgG:	-
	$F(ab')_2$:	+				

macro creatine kinase BB is due to the occurrence of specific, creatine kinase binding immunoglobulin G molecules and not due to alterations in the patients' creatine kinase BB.

2. The binding between creatine kinase BB and immunoglobulin G is specific: Three out of four of the immunoglobulin G preparations showed a high affinity to creatine kinase BB exclusively. Additionally, in one case, not only creatine kinase BB but also creatine kinase MB was bound to a great extent. Both isoenzymes showed a typical competition for the binding sites on the immunoglobulin G molecules, in the course of which creatine kinase BB revealed a higher affinity to immunoglobulin G than creatine kinase MB.

3. The Fc pieces of the IgG molecules involved in the macro creatine kinase complexes were still able to react with staphylococcal protein A without detaching the bound creatine kinase BB. We therefore assume that the Fc pieces are free i. e. they are apparently not participants in the binding between creatine kinase BB and immunoglobulin G.

4. These findings were confirmed by our results after pepsin and papain digestion of the immunoglobulin G. By exclusion chromatography, electrophoresis followed by activity staining or autoradiography and double diffusion, followed by double antibody fixation of the creatine kinase BB, we were able to localize the binding between the immunoglobulin G and creatine kinase BB in the Fab region of the immunoglobulin G. According to the increase of the molecular weight of creatine-kinase BB, due to connection with the Fab fragments of immunoglobulin G, a stoichiometric ratio of 1:1 between creatine kinase BB and the Fab fragments of IgG is highly probable.

In contrast to our results from complexing Fab fragments with creatine kinase isoenzymes, there was no comparable binding of creatine kinase BB with the Fc fragments of immunoglobulin G.

5. In two cases we were able to identify the subclass of the immunoglobulin G light chains involved in the macro creatine kinase BB. The monoclonal form of these light chains likewise indicated a specific immunological antigen-antibody reaction.

The objective of this study was to elucidate the nature of the binding between creatine kinase BB and immunoglobulin G in the sera of four patients with proven macro creatine kinase BB. *Jockers-Wretou* et. al. (12) excluded the possibility of antibody formation against creatine kinase BB, although in their reported case the macro creatine kinase proved to be composed of creatine kinase BB and immunoglobulin G. Their conclusions were drawn from experiments which, in our opinion, have only reduced probative force. Here we are able to show that a specific immunological reaction between creatine kinase BB and the Fab region of the binding immunoglobulin G caused the unusual behaviour of creatine kinase in these sera.

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